Simvastatin Reduces the Association of NMDA Receptors to Lipid Rafts
A Cholesterol-Mediated Effect in Neuroprotection

Jovita Ponce, BS; Natalia Pérez de la Ossa, MD; Olivia Hurtado, PhD; Mónica Millan, MD; Juan F. Arenillas, MD, PhD; Antonio Dávalos, MD, PhD; Teresa Gasull, PhD

Background and Purpose—Excess brain extracellular glutamate induced by cerebral ischemia leads to neuronal death, mainly through overactivation of N-methyl-D-aspartate (NMDA) receptors. The cholesterol-lowering drugs statins have been reported to protect from NMDA-induced neuronal death but, so far, the mechanism underlying this protection remains unclear. Because NMDA receptors have been reported to be associated with the cholesterol-rich membrane domains known as lipid rafts, we have investigated the effect of treatments that deplete cholesterol levels on excitotoxicity and on association of NMDA receptors to lipid rafts.

Methods—Primary neuronal cultures were pretreated with inhibitors of cholesterol synthesis and cholesterol, and NMDA-induced cell death was determined by measuring release of lactate dehydrogenase. Lipid raft fractions were isolated and Western blots were performed.

Results—Treatment with the inhibitors of cholesterol synthesis simvastatin, which inhibits the first step of cholesterol synthesis, or AY9944, which inhibits the last step of cholesterol synthesis, protected neurons from NMDA-induced neuronal death by 70% and 54%, respectively. Treatment with these compounds reduced neuronal cholesterol levels by 35% and 13%, respectively. Simvastatin and AY9944 reduced the association of the subunit 1 of NMDA receptors (NMDAR1) to lipid rafts by 42% and 21%, respectively, and did not change total expression of NMDAR1. Addition of cholesterol reduced neuroprotection by statins and AY9944, and partially reverted the effect of simvastatin on the association of NMDAR1 to lipid rafts.

Conclusions—These data demonstrate that reduction of cholesterol levels protects from NMDA-induced neuronal damage probably by reducing the association of NMDA receptors to lipid rafts. (Stroke. 2008;39:1269-1275.)

Key Words: excitotoxicity • ischemia lipids • statins

Stroke is the second cause of death worldwide and a leading cause of serious, long-term disability. Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system. However, immediately after ischemia, excessive synaptic release of glutamate, and reversal of glutamate transport increase glutamate levels in brain extracellular fluid.1,2 As a result, levels of extracellular glutamate reach concentrations that induce excessive stimulation of glutamate receptors. This overactivation of glutamate receptors drives a neuronal death called excitotoxicity that initiates with an excessive calcium entry through ionotropic glutamatergic receptors mainly of the N-methyl-D-aspartate (NMDA) subtype.3

The relevance of glutamate in the pathophysiology of tissue necrosis has been demonstrated in several experimental models of ischemia.4,5 In humans, increased glutamate levels in the extracellular fluid, and increased plasma glutamate levels have been reported to persist in some cases for >6 days after the onset of stroke.6,7 Interestingly, high levels of glutamate in plasma and cerebrospinal fluid of stroke patients on admission predict stroke progression.8 Moreover, the individual susceptibility to excitotoxic damage may be genetically determined. A recent report shows that individuals with a functional polymorphism in the promoter of the glial glutamate transporter EAAT2, which impairs its expression, show higher plasma glutamate concentrations and higher frequency of progressing stroke.9 For these individuals, targeting excitotoxic pathways after hours or even days of the initial stroke event might be important to stop progression of damage.

Recent studies have demonstrated that statins and methyl-β-cyclodextrins, compounds that deplete membrane chole-

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From Neuroscience Basic Research Lab (J.P., T.G.) and Department of Neurosciences (J.P., N.P.O., M.M., J.A., A.D., T.G.), Fundació Institut d’Investigació en Ciències de la Salut Germans Trias i Pujol, Badalona, Spain; Universitat Autònoma de Barcelona, Badalona, Spain; Departamento de Farmacología (O.H.), Facultad de Medicina, Universidad Complutense de Madrid, Madrid, Spain.
Correspondence to Teresa Gasull, Neuroscience Basic Research Lab, Fundació Institut d’Investigació en Ciències de la Salut Germans Trias i Pujol, Ctra. de Can Ruti, Camí de les escoles s/n, 08916, Badalona, Barcelona, Spain. E-mail teresagasull@yahoo.com
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terol levels, protect neurons in vitro from cell death induced by excessive stimulation of NMDA receptors. At present, the mechanism involved in statin protection against excitotoxicity is unknown. However, NMDA receptors have been reported to be associated with the cholesterol-rich membrane microdomains known as lipid rafts that bind specific proteins. Lipid rafts have been proposed to function as platforms that allow local concentration of rafts-associated proteins, promoting the interaction of protein complexes, and modulating neurotransmitter signaling. Therefore, treatment with statins might affect the functionality of proteins associated to lipid rafts, for instance, NMDA receptors.

The purpose of this study was to determine whether inhibition of cholesterol synthesis prevents neurotoxicity by NMDA and reduces the percentage of NMDA receptors associated to cholesterol-rich lipid rafts. We hypothesize that NMDA receptor signaling to death requires association of the receptor to lipid rafts.

Materials and Methods

Materials
Simvastatin sodium salt was from Calbiochem. Goat anti-NMDAR1 and rabbit anti-floplin-1 antibodies were from Santa Cruz Biotechnology. FITC-rabbit antigoat was from Zymed laboratories. All other chemicals were from either Sigma or Merck.

Primary Cultures of Neurons
Neurons were obtained as described previously. Seeded at a density of $1.5 \times 10^5$ cells/mm$^2$, and grown in Neurobasal medium supplemented with B-27, 0.5 mmol/L glutamine, and 40 $\mu$g/mL gentamycin. One-third and one-fourth of the medium was changed at 4 and 7 days in vitro, respectively.

Treatments
We used simvastatin because its high lipophilicity and blood–brain barrier permeability. For studies with statins, neurons 7 days in vitro were incubated with different concentrations of simvastatin for 4 days. For studies with AY9944, neurons were exposed to 1 $\mu$mol/L AY9944 for different times. Cholesterol was added when required. At 11 days in vitro, 100 $\mu$mol/L NMDA was added to the medium containing pretreatments, and cell death was determined 24 hours after the addition of NMDA.

Cell Death Studies
Cell death was assessed by measuring the activity of lactate dehydrogenase released from damaged cells using the lactate dehydrogenase–cytotoxicity assay kit from Biovision. In some experiments and as a second measure of cell death, percent of dead neurons was determined using propidium iodide staining (30 minutes incubation with 10 $\mu$mol/L propidium iodide followed by fixing and microscopy visualization).

Immunocytochemistry
Neurons fixed with acetone were incubated with goat anti-NMDAR1 antibody and with FITC-rabbit antigoat second antibody in the presence of Hoechst 33342. The images were obtained using a Leica TCS SP2 AOBS laser scanning confocal microscope.

Determination of Cholesterol
Cholesterol concentration was measured in neurons not exposed to NMDA using the Amplex Red Cholesterol Assay (Invitrogen) according to the manufacturer’s instructions.

Biochemical Isolation of Lipid Rafts

From Neurons
Neurons were collected in buffer A (50 mmol/L Tris-HCl, pH = 7.4, 1 mmol/L EDTA, 100 mmol/L NaCl) in the presence of 1 mmol/L PMSF and complete mini protease inhibitor mixture, homogenized, and incubated in the presence of 1% (v/v) Triton X-100 for 1 hour. Sucrose concentration was then adjusted to 45% (wt/vol) and samples were transferred to the bottom of an ultracentrifuge tube. On top of the sample, layers of 35% (3 mL), 16% (1 mL), and 5% (0.2 mL) sucrose in buffer A were added sequentially. Tubes were centrifuged at 237,000g for 18 hours in a SW 55TI rotor. The whole procedure was performed at 4°C. Once centrifuged, 9 fractions of 560 $\mu$L each were carefully collected from the top of the gradient and labeled as fraction 1 to fraction 9 following the order of collection.

SDS-Page and Western Blot Analysis

Protein concentration of samples was measured with the BCA Protein Assay kit (Pierce). To investigate total expression of the subunit 1 of NMDA receptors (NMDAR1), neurons were lysed in buffer B (62.5 mmol/L Tris pH 8.0, 10% glycerol, 2% SDS) in the presence of 1 mmol/L PMSF and complete mini protease inhibitor mixture, sonicated, and frozen until use. When used, samples were diluted in buffer B containing 1% bromophenol blue and 2.5% $\beta$-mercaptoethanol and then boiled. To perform Western blots of the raft fractions, the protein of each fraction was precipitated using the trichloroacetic acid–deoxycholate protein precipitation protocol and precipitates were resuspended in NuPAGE LDS. Electrophoresis were performed in NuPAGE Midi 10% Bis-Tris following instructions of Invitrogen. Proteins were transferred to polyvinylidene fluoride Immobilon-P Transfer Membranes, the membranes were incubated with the corresponding first antibody and horseradish peroxidase–conjugated second antibody, and immunoreactivity was detected using chemiluminescence and imaged on a Fuji medical X-ray film.

Statistical Analysis

Results are expressed as mean±SE of at least 3 different experiments. Different experiments were performed in cultured neurons obtained in different dates. Each experiment used fetuses from different pregnant rats and has usually at least 3 wells per treatment. Lipid rafts were obtained from fresh neuron homogenates pooled from several wells of 1 experiment exposed to identical treatment. Data of Westerns from lipid rafts are average of ≥3 experiments. Statistical significance of differences was determined using Student t test or 1-way ANOVA followed by Student-Newman-Keuls test.

Results

Inhibitors of Cholesterol Synthesis Simvastatin and AY9944 Protect From NMDA-Induced Neuronal Death

To answer whether inhibition of cholesterol synthesis prevents neurotoxicity by NMDA we used 2 compounds, simvastatin and AY9944, that inhibit the initial and the last step of cholesterol synthesis, respectively. By using AY9944, we inhibit cholesterol synthesis without inhibiting farnesylation or isoprenylation of proteins (Figure 1). Our primary cultures are pure neuronal cultures (<5% of non-neuronal cells) expressing NMDA receptors (Figure 2B). Exposure to 100 $\mu$mol/L NMDA for 24 hours induced neuronal death. Four days of pretreatment with simvastatin drastically reduced NMDA-induced cell death in a concentration-dependent manner as measured by lactate dehydrogenase release. The maximal effect of simvastatin was a 70% protection from NMDA-induced neurotoxicity and the EC50 with 95% CIs was 2.13 nmol/L (0.93 to 4.9 nM). Protection
by simvastatin at concentrations >250 nmol/L was not studied because concentrations of simvastatin ≥60 nmol/L already produced lactate dehydrogenase release (Figure 2B). Cell death calculated as percent of propidium iodide stained cells versus total cells was 6.6% ± 0.5, 35% ± 0.87, and 6% ± 2.3 for control, NMDA, and NMDA plus 250 nmol/L simvastatin-treated neurons, respectively.

Twelve hours of pretreatment with 1 μmol/L AY9944 prevented NMDA-induced cell death by 54%, an effect that was not improved for longer pretreatment times (Figure 3A).

**Simvastatin and AY9944 Decrease Total Cholesterol Content in Primary Cultures of Cortical Neurons**

Five-day treatment with simvastatin induced a concentration-dependent reduction of total cholesterol levels. Total cholesterol levels were reduced by 13% and 35% in neurons incubated with 31.25 nmol/L and 250 nmol/L simvastatin, respectively (Figure 2D). Reduction of cholesterol levels by 250 nmol/L simvastatin was significantly different from that induced by 31.25 nmol/L simvastatin. Twelve hours of treatment with 1 μmol/L AY9944 reduced total cholesterol content by 13%, whereas longer exposures did not reduce further cholesterol content (Figure 3B). The effect of higher concentrations of AY4499 on cholesterol levels was not studied because we found that 10 μmol/L AY9944 was toxic to neurons (not shown). Equivalent reductions of cholesterol levels (13% reduction) induced by either 31.25 nmol/L simvastatin or 1 μmol/L AY9944 produced exactly the same percentage of neuroprotection (50% reduction in excitotoxicity; Figures 2 and 3).

**Addition of Cholesterol Reduced Protection by Simvastatin and AY9944 Against Excitotoxicity**

Cholesterol is insoluble in water and is usually dissolved in ethanol to use in cell treatment. We used a complex of cholesterol with methyl-β-cyclodextrins that makes cholesterol water-soluble to prevent that ethanol stimulates NMDA-induced excitotoxicity. Addition of cholesterol reduced protection by simvastatin and AY9944 against excitotoxicity.

**Figure 1.** Biosynthesis of cholesterol in mammals. The enzyme that mediates the conversion of HMG-CoA to mevalonate and the enzymes that mediate the conversion of lanosterol to cholesterol are indicated in italics. Statins inhibit HMG-CoA reductase and thus inhibit the synthesis of geranyl and farnesyl pyrophosphate that are required to prenylate proteins. AY9944 inhibits sterol Δ14-reductase and Δ7-reductase, the last enzymes of the cholesterol biosynthetic pathway, without reducing prenylation of proteins.

**Figure 2.** (A) Fluorescence image of cells stained with propidium iodide (nonviable cells in red) merged with phase contrast microscopy images. Image on the left shows NMDA-treated neurons and image on the right NMDA-treated neurons that had been pretreated with 250 nmol/L simvastatin. (B) Concentration effect of 4-day pretreatment with simvastatin on NMDA-induced cell death measured as lactate dehydrogenase release (n=11). (C) Immunocytochemistry of fixed neurons. NMDAR1 subunits were labeled in green and nuclei in blue. Image on the right results from integration of a gallery of confocal images. Scale bars=10 μm. (D) Effect of 5-day treatment with simvastatin on neuronal cholesterol content (n=8). *Significantly different from control and treated with simvastatin (P<0.05), #Significantly different from control cells (P<0.05), $Significantly different from control cells treated with NMDA and not pretreated with simvastatin (P<0.05).
experiments, cyclodextrins are devoid of any effect on excitotoxicity and, if any, cyclodextrins should protect against NMDA-induced neuronal death. However, we found that the addition of 10 μmol/L cholesterol partially prevented statin-induced neuroprotection and fully prevented AY9944-induced protection against excitotoxicity (Figure 4).

Simvastatin and AY9944 Do Not Change the Expression of NMDA Receptors
To reject that protection by either simvastatin or AY9944 in front of excitotoxicity could be associated with reduced expression of the NMDA receptor, we investigated the effect of both compounds on NMDA expression. We found that even 4 days of treatment with these compounds did not change the expression of NMDAR1 (Figure 5).

Characterization of the Protocol to Obtain Lipid Rafts
After the ultracentrifugation of the samples on a sucrose gradient, we obtained 9 fractions. Lipid rafts were tracked in the fractions by the enrichment on cholesterol and the raft marker flotillin. Cholesterol and flotillin were concentrated between fractions 1 and 5, peaking in fraction 2. In contrast, protein and the nonlipid raft protein actin were concentrated in fractions 8 and 9 (Figure 6A). For each experiment, we analyzed by Western blot the profile of immunoreactivity for NMDAR1 in each of the 9 fractions obtained. After densitometry, we added results obtained for each of the fractions belonging to lipid rafts, and calculated percent of receptors present in rafts versus total receptors. In control neurons, 60% of the immunoreactivity for the NMDAR1 subunit was usually found associated to lipid rafts.

Simvastatin and AY9944 Reduce, and Cholesterol Partially Restores, the Association of NMDAR1 Receptors to Lipid Raft Fractions
Four days of treatment with simvastatin 250 nmol/L, or 8 hours of treatment with AY9944 1 μmol/L, reduced the presence of NMDAR1 in the fractions corresponding to lipid rafts by 42% and 21%, respectively (Figure 6C). Furthermore, the addition of cholesterol partially reverted the effect produced by statins in the association of the NMDA receptor to lipid rafts (Figure 6C).

Discussion
Our results support the hypothesis that cholesterol status of neurons is crucial for excitotoxicity since we found that inhibition of cholesterol synthesis prevents NMDA-induced neuronal death. Two different inhibitors of cholesterol synthesis, the inhibitor of 14-Δ7 reductase AY9944 and the inhibitor of HMGCoA reductase simvastatin, protect from excitotoxic cell death in pure neuronal cultures (Figures 2 and 3). Both compounds inhibited cholesterol synthesis, but contrary to simvastatin, AY4499 does not inhibit isoprenyla-
tion or farnesylation. In agreement with results previously reported, we found that simvastatin protected against excitotoxicity at nanomolar concentrations and required pretreatments 2 days to provide neuroprotection. These 2 observations suggest that the effect of simvastatin on neuroprotection relays on its effect on cholesterol synthesis, because the inhibition of prenylation by statins occurs at higher concentrations and with shorter exposure times than those required to obtain neuroprotection.

The 2 previous reports demonstrating protection of excitotoxicity by statins do not agree each other about the importance of the inhibition of cholesterol synthesis for statin-induced neuroprotection. The discrepancies were explained by differences in the experimental model, because the second work concluded that neuroprotection by statins was not mediated by cholesterol and used pure neuronal cultures, whereas the first one concluded the opposite and used mixed neuronal–glial cultures. However, our results support that neuroprotection arises from sustained inhibition of cholesterol synthesis even when using pure neuronal cortical cultures, because we report for the first time to our knowledge that pretreatment with the inhibitor of cholesterol synthesis AY9944 also protects from excitotoxicity. In addition, in our experience, cholesterol partially prevented neuroprotection by simvastatin (Figure 4A) and reverted protection by AY9944 (Figure 4B). Altogether these results indicate that cholesterol depletion is the cause of the neuroprotection afforded by inhibitors of cholesterol synthesis such as statins in front of excitotoxicity in our model. In this regard, results from a previous report, and our own (not shown), demonstrated that a compound that binds and removes membrane cholesterol, methyl-β-cyclodextrin, inhibits neuronal death induced by oxygen and glucose deprivation and also direct excitotoxic cell death, thus further supporting the hypothesis that cholesterol depletion protects from excitotoxicity.

We found that even small reductions (~13% reduction) on cholesterol levels were associated to reductions in excitotoxicity (50% reduction) independently of whether the effects of cholesterol synthesis even when using pure neuronal cortical cultures, because we report for the first time to our knowledge that pretreatment with the inhibitor of cholesterol synthesis AY9944 also protects from excitotoxicity. In addition, in our experience, cholesterol partially prevented neuroprotection by simvastatin (Figure 4A) and reverted protection by AY9944 (Figure 4B). Altogether these results indicate that cholesterol depletion is the cause of the neuroprotection afforded by inhibitors of cholesterol synthesis such as statins in front of excitotoxicity in our model. In this regard, results from a previous report, and our own (not shown), demonstrated that a compound that binds and removes membrane cholesterol, methyl-β-cyclodextrin, inhibits neuronal death induced by oxygen and glucose deprivation and also direct excitotoxic cell death, thus further supporting the hypothesis that cholesterol depletion protects from excitotoxicity.

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We found that even small reductions (~13% reduction) on cholesterol levels were associated to reductions in excitotoxicity (50% reduction) independently of whether the effects
were induced by simvastatin or AY9944 (Figures 2 and 3). Because changes in brain cholesterol metabolism have been reported in experimental animals and also in humans, sometimes with short-lasting statin treatments, it is possible that statins affect neuronal cholesterol levels soon after the beginning of treatment. In contrast with other tissues, cholesterol present in brain is entirely synthesized in situ. In addition, most cholesterol in brain is present in oligodendrocytes, and a nonsignificant reduction of total brain cholesterol might mask a large change in neuronal cholesterol.

To better evaluate the putative therapeutic role of statins in preventing excitotoxicity, we next investigated the mechanism underlying this protection. Reduction on the availability of NMDA receptors to interact with NMDA or its physiological ligand glutamate is one mechanism that might mediate protection by statins. Reduced available receptors might result from internalization of receptors, a fast mechanism, from reduced receptor expression, or from the effect of reduced membrane cholesterol on the functionality of NMDA receptors. The last 2 mechanisms fit better with the requirement of long pretreatment with simvastatin to obtain neuroprotection. However, we found that expression of NMDAR1 was unchanged after sustained treatment with either simvastatin or AY9944 (Figure 5), thus rejecting that neuroprotection would result from reduced expression of NMDA receptors.

Another mechanism by which statins might confer neuroprotection might be reduction of the association of NMDA receptors to lipid rafts. It has been reported previously that >50% of NMDA receptors are associated to lipid rafts. We demonstrated here for the first time to our knowledge that simvastatin and AY9944 reduced the association of NMDA receptors to lipid rafts and that addition of cholesterol partially curtails this effect in simvastatin-treated neurons (Figure 6). In fact, cholesterol curtails to a similar extent both the reduction on the association of NMDAR1 to lipid rafts and the neuroprotection induced by simvastatin, suggesting that both effects are related. In this regard, it has been reported that after experimental ischemia in the rat, the levels of NMDA receptor were reduced in lipid rafts. This might be a strategy to reduce neurodegeneration by ischemia. If our hypothesis is correct, statin-induced removal of NMDA receptors from rafts might provide neurons with best probabilities to survive after ischemia-reperfusion.

Statins are currently used to prevent stroke recurrence, and recent studies suggest that statin pretreatment improves stroke outcome, and that cessation of statin treatment in stroke patients that had been chronically treated with statins before the stroke event is associated with poor outcome. Moreover, statins have been reported to be neuroprotective in animal models of brain ischemia even when administered after the induction of ischemia. Thus, statins not only effectively prevent acute stroke but also might be of potential benefit to treat it. The mechanism by which statins provide benefit against stroke seems to be multifactorial and has been related to the effects of statins on the improvement of endothelial function through increased endothelial nitric oxide synthase, on the regulation of the fibrinolytic balance, and on antioxidant mechanisms. However, taking into account that at least 3 different experimental approximations impairing membrane cholesterol content and reducing the association of NMDAR to lipid rafts (Figure 6) proved protective against excitotoxicity and oxygen glucose deprivation in cultured neurons (Figures 2 and 3), inhibition of excitotoxicity by statins in the acute phase might be one of the multiple mechanisms that provide neuroprotection by statins in ischemia. This mechanism might be particularly relevant to groups of patients prone to develop progressive stroke as a result of its genetically driven impossibility to remove glutamate from extracellular fluid during stroke. In humans, the possibility that statins could be used for the treatment of acute ischemic stroke has not yet been addressed. However, in rats proved beneficial in a combined treatment with tissue plasminogen activator. Therefore, while awaiting the studies in humans to confirm the benefit of treatment with statins or statins plus tissue plasminogen activator in the acute phase of stroke, the study of the mechanisms of action of statins, both pleiotropic and mediated by its cholesterol-lowering properties, might help to design an efficient statin therapy.

Because statins affect cholesterol-containing lipid rafts, it is important to consider that the glial glutamate transporter associates also to lipid rafts, and that cholesterol depletion has been reported to increase by 50% its glutamate uptake activity even though reduces the activity of the glial glutamate inactivating enzyme glutamine synthetase. The effect of increased glutamate uptake by cholesterol-depleted astrocytes in neuronal death during ischemia has not been studied so far. In culture, cholesterol-depleted astrocytes were more vulnerable to glutamate-induced death, but this excitotoxic effect was observed at concentrations 8-fold higher than the maximum glutamate concentrations found in extracellular fluid in massive stroke conditions. Therefore, in pathophysiological conditions cholesterol depletion might improve glutamate uptake without showing toxicity to astrocytes. This effect might also contribute to neuroprotection by statins in the acute phase of stroke.

This study asked whether cholesterol levels and association of NMDA receptors to lipid rafts are important for NMDA signaling to death. We concluded that reduction in cholesterol associates with protection from excitotoxicity, and that cholesterol depletion and, thus, statins, might prevent in vivo neuronal cell death during ischemia by directly preventing the damaging effects of the overactivation of NMDA receptors associated to lipid rafts. However, the dose–response effect of simvastatin and of cholesterol addition on the association of NMDA receptors to lipid rafts should be addressed in future experiments. In addition, our results suggest that AY9944, which reduces cholesterol levels and prevents excitotoxicity much faster than statins, might be a promising new compound to protect against cerebral ischemia.

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